with asthma [18], but few studies have examined the association of ADS exposure with upper respiratory tract symptoms in adult asthma.

In animal models, airborne particles originating from ADSs in Mongolia and northern China have been shown to increase pulmonary inflammation and infiltration [19–21]. The mechanism is unclear, but Sierra-Vargas et al. showed that neutrophils migrate to the lung during acute inflammation induced by exposure to air pollutants [22]. Air pollutants also increase the concentration of interleukin (IL)-8 in bronchial lavage fluid (BALF) and IL-8 mRNA expression in bronchial biopsy tissue obtained from healthy subjects [23]. IL-8 increases in the blood of asthma patients during exacerbation [24], and thus is thought to be a key cytokine in exacerbation of asthma.

In this study, we investigated the relationship between exposure to an ADS and upper and lower respiratory tract symptoms and respiratory function in adult patients with controlled asthma. An IL-8 luciferase assay in a stable THP-1-derived IL-8 reporter cell line was used to investigate the effect of airborne ADS particles.

Materials and methods

Patients

A total of 184 outpatients aged >18 years old with asthma were recruited into the study from December 2010 to January 2011. The patients were residents in four different locations, Yonago City, Matsue City, Toyooka City, and Kanda Town, which are distributed in a rural area of under 200 km diameter in western Japan. Of these patients, 112 were included in the analysis based on the following inclusion criteria (Figure 1): (1) mild to moderate asthma, as defined by the National Heart, Lung, and Blood Institute [25], (2) controlled asthma within 3 months before March 2011, based on the Global Initiative for Asthma (GINA) definition [26], (3) recorded daily respiratory symptoms and PEF for >90% of days from March to May, 2011, and (4) no hospitalization except for asthma from March to May, 2011. On the basis of GINA criteria, asthma was defined as positive if a case met (1) and (2) or (3) of the following criteria: (1) a history of intermittent wheezing; (2) airway hyper-responsiveness to methacholine; and (3) reversible airflow limitation (12% and 200 ml variability in FEV_1). The Research Ethics Committees of each participating institution approved the study and all patients gave written informed consent.

Definition of the period of ADS exposure and monitoring of air pollutants

The period of ADS exposure was determined using information from the Japan Meteorological Agency based on a criterion of visibility <10 km due to dust arising from the deserts of East Asia, as determined by meteorological satellites. Concentrations of suspended particular matter (SPM), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), and photochemical oxidants (O_x) were confirmed based on monitoring at many locations in Japan by the Japanese Ministry of the Environment. In this study, we used these data for concentrations of SPM, SO_2, NO_2, and O_x at the four study locations.

Recording of daily upper and lower respiratory tract symptoms and PEF

From February to May 2011, all patients recorded their daily respiratory tract symptoms and measured their morning PEF using a peak flow meter (Mini-Wright, Harlow, England, American Thoracic Society scale). February was the practice period. Scores for upper respiratory tract symptoms such as stuffiness, sneezing, and lower respiratory tract symptoms such as cough, sputum, dyspnea, and wheezing were recorded in a diary using 0 = absent, 1 = mild, and 2 = severe for each

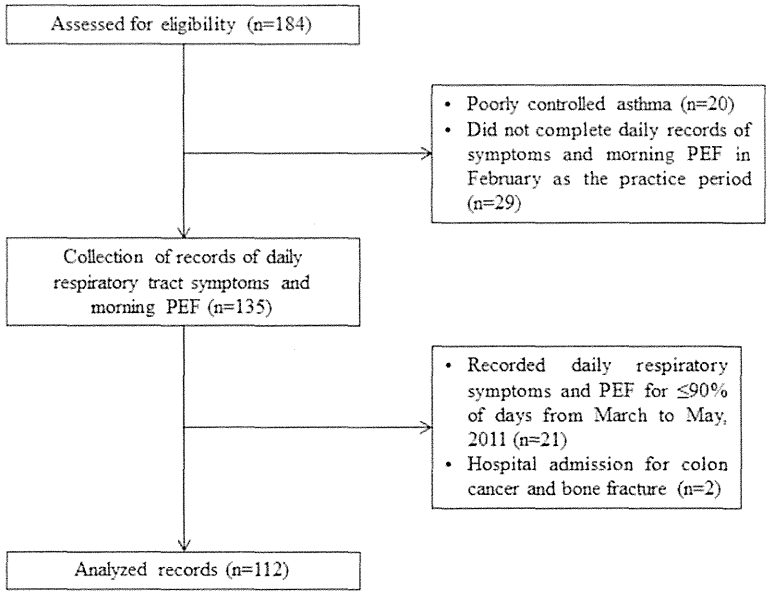

Figure 1. Flowchart showing the disposition of subjects in the study.

symptom. Unscheduled hospital visits, fever and pharyngeal pain, and use of oral corticosteroids for exacerbation of asthma on ADS days were also recorded by the patients. Respiratory tract infection was defined as being present if patients had pharyngeal pain and/or fever, which was defined as a body temperature >37.5 °C.

The total scores for each upper and lower respiratory tract symptom was calculated by summing the score for each day. The period of ADS exposure was determined to be 1–3 May 2011 using information from the Japanese Ministry of the Environment. The results were analyzed over a period of one month because climate conditions differ markedly between March and May in Japan. Thus, the control period of non-ADS exposure was defined as 10–30 April 2011. Patients with worsened symptoms associated with ADS were defined as those with wheezing or a severe score for more than two symptoms out of cough, sputum, and dyspnea without pharyngeal pain and/or fever during the ADS period. Patients recorded the best PEF value from three attempts within 30 min of waking up and before taking inhaled corticosteroids (ICS), β_2-agonists or oral drugs. The minimum morning PEF (% recent best) was determined based on the lowest daily morning prebronchodilator PEF [27]. The recent best value is defined as the best PEF from February to May 2011 in each patient. The lowest PEF values were also determined for the ADS (1 to 3 May 2011) and non-ADS (10 to 30 April 2011) periods.

Preparation of airborne particles collected on ADS days

Soil from the China Loess Plateau (CJ-1), the original ADS soil in the Tengger Desert and Huining located in Gansu Province, was obtained from the National Institute for Environmental Studies (Ibaraki, Japan) in 2002. This reference material was certified by the National Institute for Environmental Studies and the National Research Center for Environmental Analysis and Measurement (Beijing, China). Dust particles were collected in Yonago City on ADS days (1–3 May 2011) using a large acrylic basin with a collection area of 5000 cm^2 and a depth of 30 cm (custom made by Denyo Inc., Tokyo, Japan). CJ-1 and the collected dust were sterilized at 121 °C for 30 min in an autoclave (Tomy SX-300; Tomy Co., Tokyo, Japan) and stored in a freezer at −20 °C to prevent growth of bacteria and fungi. For stimulation of THP-G8 cells, airborne particles collected on ADS days were diluted to various concentrations with distilled deionized water. Supernatant extracted from water-soluble airborne particles was also collected after the particles were kept on ice for 1 h.

IL-8 promoter-luciferase gene reporter assay

THP-G8 cells are a THP-1-derived reporter cell line that express stable luciferase orange (SLO) and stable luciferase red (SLR) genes under the control of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase promoters, respectively [28]. The THP-G8 cell line was kindly provided by the Department of Dermatology, Tohoku University, Graduate School of Medicine (Sendai, Japan) and was cultured according to a previous report [28]. To measure the changes of SLO and SLR luciferase activity after exposure to CJ-1 soil and ADS airborne particles, THP-G8 cells (5×10^4 cells/100 µl/well) in 96-well black plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were stimulated for 5 h with solvent only (negative control), 100 ng/ml lipopolysaccharide (LPS) (Wako Pure Chemicals, Osaka, Japan), and various concentrations of CJ-1 soil and ADS airborne particles. The IL-8 transcriptional activity of THP-G8 cells was evaluated at maximum induction after stimulation by 100 ng/ml LPS for 5 h [28]. Luciferase activity was determined using a microplate luminometer with a Phelios multicolor detection system (Atto Co., Tokyo, Japan) using Tripluc luciferase assay reagent (Toyobo Co., Osaka, Japan). IL-8 transcriptional activity was assessed from normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA, and the fold induction of nSLO-LA was calculated as the nSLO-LA level of treated cells divided by that of untreated cells [28].

Measurement of concentrations of IL-8 and endotoxin

IL-8 concentrations in culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit for IL-8 (R&D Systems, Minneapolis, MN, USA). Samples were run in triplicate and read using an automated ELISA reader (Bio-Rad Model 680, Bio-Rad, Philadelphia, PA). The range of the assay was 31.2 to 2000 pg/ml. The endotoxin concentration in ADS airborne particles was measured with a Chromogenic LAL endotoxin assay kit (GenScript, Piscataway, NJ, USA). The range of the assay was 0.01 to 1 EU/ml. The pH of ADS airborne particles was measured with a pH meter (MP220; Mettler Toledo, Schwerzenbach, Switzerland).

Statistical analysis

Results are shown as the mean ± standard deviation (SD). SPSS Statistics software (Japanese ver. 16.0 for Windows; IBM Japan, Tokyo, Japan) was used for statistical analysis. A Mann–Whitney U test was used for comparison of air pollution data between the ADS and non-ADS periods. A χ^2 test was used for comparison of categorical data between patients with and without comorbid allergic rhinitis and chronic sinusitis. Pulmonary function in these two groups was analyzed by Mann–Whitney *U* test. Comparisons of the average score for daily upper and lower respiratory tract symptoms between the ADS and non-ADS periods were analyzed by Wilcoxon signed rank test. The difference in average PEF in the ADS and non-ADS periods was analyzed by *t*-test. Significance was defined as $p < 0.05$ in all analyses.

Results

Patient characteristics

A flowchart showing the progression of subjects through the study is shown in Figure 1. Of the initial 184 outpatients with asthma, 20 were excluded due to poorly controlled asthma and 29 declined to record daily respiratory symptoms and morning PEF during the practice period in February. Of the

remaining 135 patients, 21 did not record daily respiratory symptoms and morning PEF for >90% of days from March to May and 2 were excluded due to hospital admissions for colon cancer and bone fracture. This left 112 patients with asthma who were eligible for analysis. The characteristics of these patients are shown in Table 1. Of the 112 patients, 31 (28%) were also diagnosed with allergic rhinitis and/or chronic sinusitis by an otolaryngologist. There was a significant difference in age between patients with and without comorbid allergic rhinitis and/or chronic sinusitis. There was no difference in sex, smoking status, dose of ICS, and prevalence of treatment with long-acting β_2-agonists between the two groups.

Air pollution on ADS and non-ADS days

In the 2011 season, ADS exposure occurred from May 1 to 3 in the four locations in the study, which are located within a radius of 200 km in a rural area of western Japan. A control non-ADS period was defined from 10–30 April 2011, just before the ADS period. The daily average levels of air pollutants in the ADS and non-ADS periods in each location are shown in Table 2. The average and daily maximum SPM differed significantly between ADS and non-ADS days in the four locations. In contrast, there were no significant differences in the levels of SO_2, NO_2, and O_x between the ADS and non-ADS days in any of the locations.

Table 1. Characteristics of patients with or without comorbid allergic rhinitis (AR) and/or chronic sinusitis (CS).

	All patients	Patients without comorbid AR and/or CS	Patients with comorbid AR and/or CS
Number	112	81	31
Age (year)	61.4 ± 16.3	64.3 ± 14.7*	55.5 ± 18.0*
Gender (male/female)	43/69	31/50	12/19
Allergic rhinitis/Chronic sinusitis/Both	23/5/3	0/0/0	23/5/3
Allergic conjunctivitis	3	1	2
Atopic dermatitis	4	2	2
Smoking status Never/Ex/Current	101/8/3	72/6/3	29/2/0
FVC (L)	3.09 ± 0.91	3.00 ± 0.86	3.35 ± 0.96
FEV_1 (L)	2.45 ± 0.80	2.34 ± 0.75	2.66 ± 0.86
$\%FEV_1$ (%)	110.5 ± 18.5	106.4 ± 17.7	112.0 ± 23.5
$FEV_1\%$ (%)	79.2 ± 7.4	78.6 ± 6.7	79.1 ± 8.7
Inhaled corticosteroid	111	80	31
Low dose	27	15	12
Medium dose	73	60	13
High dose	11	5	6
Long-acting β_2-agonists	42	32	10
Leukotriene receptor antagonist	60	36	24
Theophylline	24	21	3
Histamine antagonist	26	1	25
Intranasal corticosteroid	12	0	12

Data is presented as the mean ± S.D.
FEV1; forced expiratory volume in 1 s, %FEV1; percentage of predicted FEV1, FVC; forced vital capacity.
*$p < 0.05$ by Mann–Whitney U for comparison of patients with and without comorbid allergic rhinitis and/or chronic sinusitis

Table 2. Daily average of air pollution levels in four locations during the ADS days (1–3 May 2011) and non-ADS days (10–30 April 2011).

	Yonago City		Matsue City	
	ADS days	Non-ADS days	ADS days	Non-ADS days
Av SPM, $\mu g/m^3$	80.3 ± 29.1*	19.2 ± 1.5	64.0 ± 28.2*	17.2 ± 1.5
Av daily maximum SPM, $\mu g/m^3$	124.3 ± 2.1*	38.3 ± 4.3	92.3 ± 13.8*	29.4 ± 2.4
Av SO_2, ppb	1.0 ± 0.0	1.2 ± 0.1	0.6 ± 0.1	1.0 ± 0.1
Av daily maximum SO_2, ppb	2.0 ± 0.0	2.6 ± 0.4	1.1 ± 0.2	2.1 ± 0.3
Av NO_2, ppb	6.3 ± 3.1	6.1 ± 2.0	2.0 ± 1.1	2.5 ± 1.2
Av daily maximum NO_2, ppb	13.3 ± 8.5	15.3 ± 7.4	3.5 ± 1.9	5.9 ± 3.4
Av Ox, ppb	37.7 ± 5.0	38.3 ± 8.1	46.1 ± 12.4	53.1 ± 8.0
Av daily maximum Ox, ppb	52.3 ± 2.1	52.2 ± 13.3	61.5 ± 6.5	69.1 ± 11.6
	Toyooka City		Kanda Town	
Av SPM, $\mu g/m^3$	100.6 ± 6.5*	22.2 ± 2.4	109.0 ± 35.8*	28.8 ± 11.4
Av daily maximum SPM, $\mu g/m^3$	162 ± 14.9*	32.0 ± 11.8	160.0 ± 17.8*	57.6 ± 33.2
Av SO_2, ppb	0.30 ± 0.06	0.71 ± 0.90	1.3 ± 0.6	2.1 ± 1.2
Av daily maximum SO_2, ppb	0.67 ± 0.33	1.3 ± 1.2	3.7 ± 2.1	5.9 ± 4.1
Av NO_2, ppb	3.86 ± 0.28	4.76 ± 0.48	10.0 ± 8.7	15.5 ± 10.7
Av daily maximum NO_2, ppb	13.0 ± 2.1	11.6 ± 3.7	26.0 ± 17.6	39.2 ± 22.4
Av Ox, ppb	45.2 ± 6.5	58.5 ± 2.1	37.0 ± 11.4	42.5 ± 14.4
Av daily maximum Ox, ppb	59.3 ± 6.4	67.0 ± 10.8	52.7 ± 13.7	63.8 ± 13.2

Data is presented as the mean ± S.D., Av; average, *$p < 0.05$, ADS days versus non-ADS days.

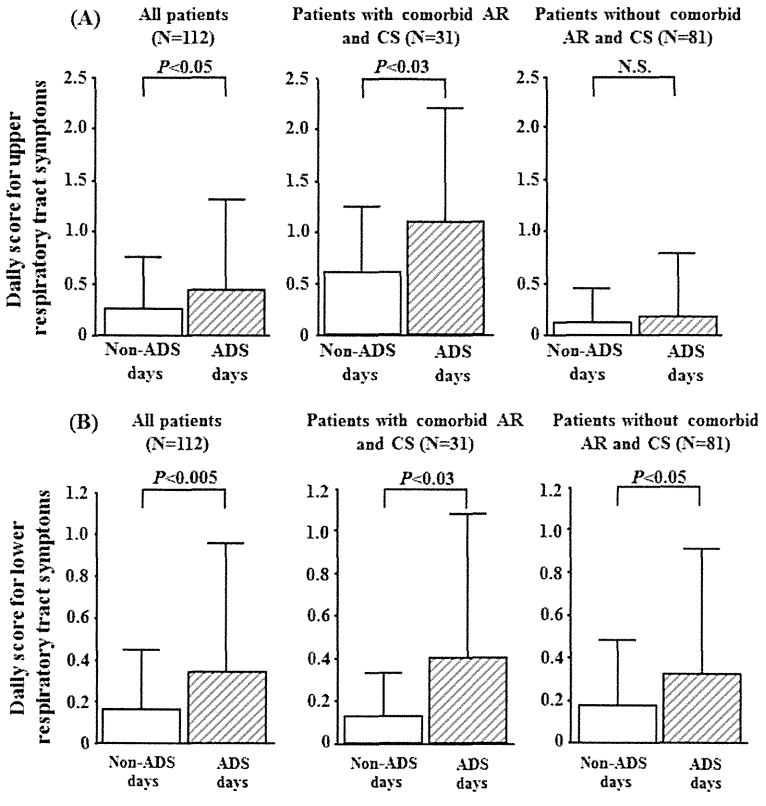

Figure 2. Scores for upper (A) and lower (B) respiratory tract symptoms in periods without (10–30 April 2011) and with (10–3 May 2011) an Asian Dust Storm (ADS) in all patients, and in patients with and without comorbid allergic rhinitis (AR) and/or chronic sinusitis (CS). Scores for upper respiratory tract symptoms such as stuffiness, sneezing, and lower respiratory tract symptoms such as cough, sputum, dyspnea, and wheezing were defined as 0 = absent, 1 = mild, and 2 = severe for each symptom.

Respiratory symptom scores and PEF

In all patients, scores for upper respiratory tract symptoms were significantly higher on ADS days compared to non-ADS days (0.45 ± 0.08 vs. 0.26 ± 0.05, $p < 0.05$; Figure 2A). Similarly, patients with comorbid allergic rhinitis and/or chronic sinusitis had significantly increased scores for upper respiratory tract symptoms on ADS days compared to non-ADS days (Figure 2A). In contrast, in patients without comorbid allergic rhinitis and/or chronic sinusitis, there was no significant difference in these scores on ADS and non-ADS days.

Scores for lower respiratory tract symptoms in all patients were also significantly higher on ADS days compared to non-ADS days (0.35 ± 0.61 vs. 0.16 ± 0.28, $p < 0.005$; Figure 2B). There were significant differences in the scores for these symptoms between ADS and non-ADS days in patients with and without comorbid allergic rhinitis and chronic sinusitis (Figure 2B). Three patients made unscheduled hospital visits and took oral corticosteroids due to exacerbation of asthma without respiratory tract infection during the ADS period. In contrast, no patients needed oral steroids for exacerbation of asthma on non-ADS days, except for one event of exacerbation of asthma due to respiratory tract infection.

The mean morning PEF values were 384.9 ± 89.6 and 382.3 ± 90.2 l/min on ADS and non-ADS days (Figure 3A), with no significant difference between the periods. There were also no significant differences in mean morning PEF on ADS days compared to non-ADS days in patients with and without comorbid allergic rhinitis and/or chronic sinusitis (Figure 3A). The respective minimum morning PEF values were $92.4 \pm 5.9\%$ and $92.7 \pm 4.6\%$ in all patients, $93.4 \pm 3.2\%$ and $93.2 \pm 4.2\%$ in patients with comorbid allergic rhinitis and/or chronic sinusitis, and $92.3 \pm 5.1\%$ and $92.0 \pm 6.4\%$ in patients without comorbid allergic rhinitis and/or chronic sinusitis (Figure 3B), also without a significant difference.

IL-8 transcriptional activity and IL-8 secretion in THP-G8 cells

The pH values of ADS airborne particles (400 μg/ml) and CJ-1 soil (400 μg/ml) were 7.9 and 8.4, respectively. THP-G8 cells were stimulated with CJ-1 soil after adjustment of the pH of the soil to 7.8 with 0.1 N sodium hydroxide. nSLO-LA was not increased before adjusting the pH (data not shown). Exposure of pH-adjusted CJ-1 soil ($n = 6$) also did not increase nSLO-LA in THP-G8 cells (Figure 4). In contrast, nSLO-LA was increased in exposure of cells to ADS airborne particles ($n = 6$) in a dose-dependent manner (Figure 5). Stimulation of THP-G8 cells with supernatant of ADS airborne particles caused nSLO-LA to increase to 1.3 ± 0.1 at 100 μg/ml and to 3.1 ± 0.2 at 400 μg/ml (Figure 5). However, the increase of nSLO-LA was smaller with the supernatant compared with the ADS airborne particles. The concentrations of IL-8 in supernatants of THP-G8 cells stimulated with vehicle, LPS ($n = 6$, 100 ng/ml), CJ-1 soil ($n = 6$, 400 μg/ml) and ADS airborne particles ($n = 6$,

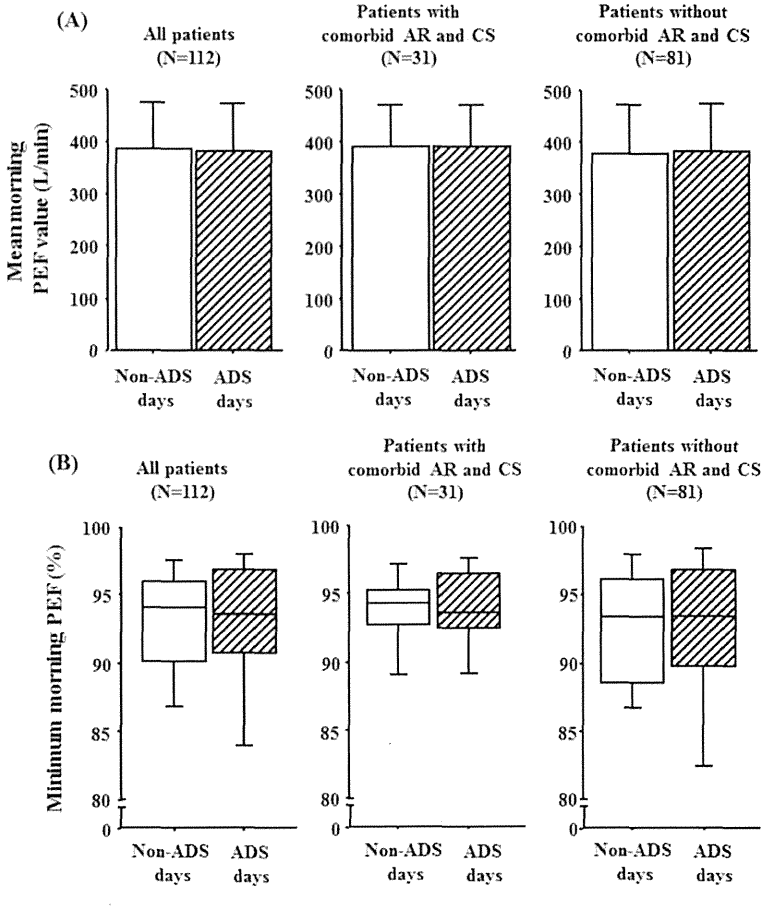

Figure 3. Morning peak expiratory flow (PEF) (A) and minimum morning PEF (% recent best) (B) in a period without (10–30 April 2011) and with (1–3 May 2011) an Asian Dust Storm (ADS) in all patients, and in patients with and without comorbid allergic rhinitis (AR) and/or chronic sinusitis (CS). The best PEF value was that recorded in three attempts within 30 min of waking up and before taking inhaled corticosteroids (ICS), β_2-agonists or oral drugs. The minimum morning PEF (% recent best) is defined as the lowest daily morning prebronchodilator PEF [26].

400 μg/ml) were 0.03 ± 0.002, 38.1 ± 1.7, 0.30 ± 0.004, and 29.7 ± 1.5 μg/ml, respectively (Figure 5).

Endotoxin concentration in airborne particles collected on ADS days

The endotoxin concentrations were 0.192 EU/ml in ADS airborne particles at 400 μg/ml and 0.074 EU/ml in CJ-1 soil at 400 μg/ml. However, LPS at 100 ng/ml was out of the range of the assay, so we measured at 100 pg/ml of LPS. The endotoxin concentration was 0.885 EU/ml.

Discussion

This study showed that exposure to an ADS aggravated upper and lower respiratory tract symptoms in adult patients with controlled asthma, with three patients having unscheduled hospital visits for exacerbation of asthma without respiratory tract infections on ADS days. IL-8 transcriptional activity in THP-G8 cells was stimulated by airborne particles collected on ADS days. These results suggest that asthma might be exacerbated by ADS exposure due to enhanced airway inflammation mediated by increased IL-8 in the airway.

Our previous telephone surveys showed that exposure to an ADS can aggravate lower respiratory tract symptoms and respiratory function in asthma patients [14,15]. In this study, we assessed both lower and upper respiratory tract symptoms on ADS and non-ADS days, and found that adult patients with asthma had worsened upper and lower respiratory tract symptom scores on ADS days. Three patients also had unscheduled hospital visits for asthma, although none were hospitalized, suggesting that ADS exposure has effects on symptoms in patients with controlled asthma. The worsening of upper respiratory tract symptoms in patient with asthma, and especially in those with comorbid allergic rhinitis and/or chronic sinusitis, on ADS days, is consistent with the findings in Min et al. [18]. In the present study, there were no significant differences in the mean morning PEF and the minimum morning PEF (% recent best) between ADS and non-ADS days, despite the worsening of symptoms. The lack of detection of a decrease in PEF on ADS days may have been due to the lower average level of SPM on these days in 2011 compared to our previous studies [14,15]. Alternatively, peak flow may not be a sensitive parameter for detecting subtle changes in asthma [29] and the patients in this study had well-controlled asthma.

IL-8 is a key chemokine in airway inflammation induced by air pollutants [23,24] and Honda et al. found that components adhered to ASD can increase release of IL-6 and IL-8 from airway epithelial cells [30]. An IL-8 promoter luciferase assay in THP-G8 cells, the so-called IL-8 Luc

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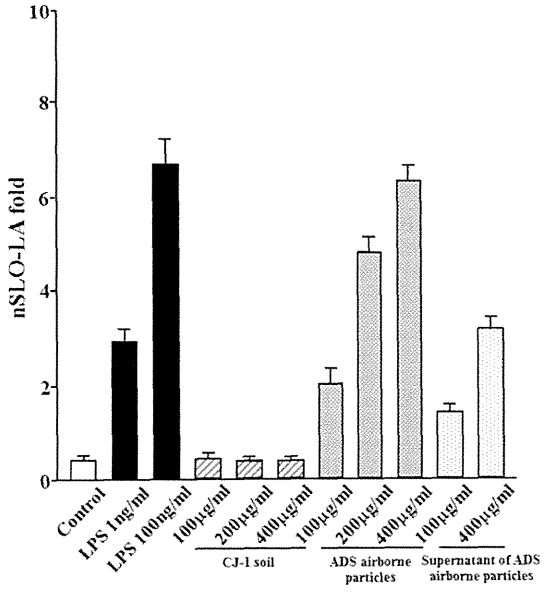

Figure 4. IL-8 transcriptional activity measured using an IL-8 luciferase assay in a stable THP-1-derived IL-8 reporter cell line. Cells were treated with solvent only (negative control), LPS (positive control), CJ-1 soil, airborne particles, and supernatant extracted from airborne particles collected during an ADS. IL-8 transcriptional activity was assessed from normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA. The fold induction of nSLO-LA was calculated as the nSLO-LA of treated cells divided by that of untreated cells [27].

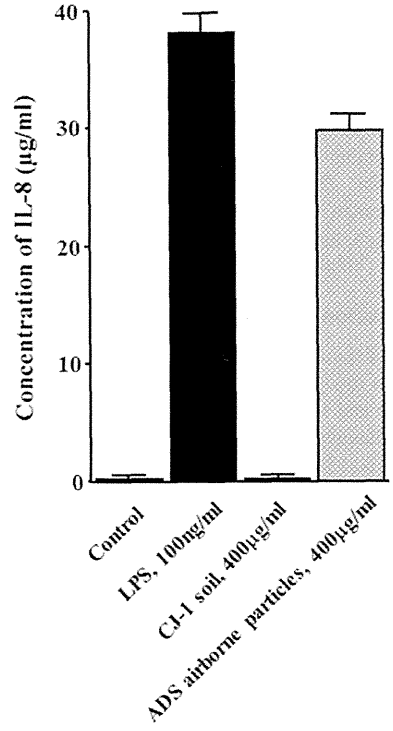

Figure 5. Concentration of IL-8 in supernatants of a stable THP-1-derived IL-8 reporter cell line stimulated with solvent only (negative control), LPS ($n=6$, 100 ng/ml), CJ-1 ($n=6$, 400 μg/ml), and dust particles collected during an ADS ($n=6$, 400 μg/ml). The concentration of IL-8 was measured using an ELISA kit. Samples were run in triplicate. The range of the assay was from 31.2 to 2000 pg/ml.

assay, was used in the study because it has high sensitivity for evaluation of the IL-8 level using a small amount of material. Sand dust particles of ADS in Japan contain various chemicals, metals, microorganisms and ionic components [31,32]. To examine exacerbation of respiratory function by sand dust particles, we measured the difference in production of IL-8 between ADS airborne particles and the origin CJ-1 soil of the ADS. We found that ADS airborne particles promoted transcriptional activity and production of IL-8 in THP-G8 cells, with a significant increase in IL-8 transcriptional activity in THP-G8 cells treated with ADS airborne particles compared to those treated with original ADS soil. This difference may be caused by substances such as chemicals, metals and microorganisms carried by the ADS, in agreement with the findings of Honda et al. [30].

A large amount of precipitate was formed when an aqueous solution of airborne particles collected on ADS days was kept on ice for 1 h. The supernatant containing water-soluble ADS airborne particles also augmented IL-8 transcriptional activity in THP-G8 cells. This suggests that materials attached to or contained in the soil augmented this activity. Thus, ADS particles may exacerbate aggravation of asthma when chemicals, metals and microorganisms adhere to these particles.

Patients with asthma are more sensitive to the effects of environmental endotoxins (LPS) compared to healthy subjects, and inhaled LPS is associated with airway neutrophil inflammation in patients with asthma and in healthy subjects [33,34]. Our results showed that ADS airborne particles contained endotoxin, as also found previously [32], but the original soil did not do so, which suggests that endotoxin might have contributed to augmentation of the IL-8 transcriptional activity in THP-G8 cells.

There are several limitations in the study. First, we were not able to collect the same amount of airborne particles on non-ADS days because the level of SPM was very low on these days. Therefore, we were unable to compare stimulation of IL-8 transcription by airborne particles on ADS days with that by particles collected on non-ADS days. Second, we were not able to analyze the composition of airborne particles and the supernatant. Third, we did not assess the amount of pollen, which can affect upper respiratory tract symptoms in rhinitis patients from January to March, distinct from the period of ADS exposure. Future work is required to collect more airborne particles using two high-volume air samplers and a classification of the particles into four size ranges.

Conclusion

We found that upper and lower respiratory tract symptoms in adult asthma patients worsened during a period of exposure to an ADS. Airborne particles collected on ADS days in western Japan, but not the origin soil of the ADS, increased IL-8 secretion in THP-G8 cells. This finding suggests that

exposure to an ADS may exacerbate asthma by enhancement of IL-8 transcriptional activity. Further studies are needed to better define the association between asthma and ADS.

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Declaration of interest

None of the authors have a conflict of interest regarding the work in this study. The authors alone are responsible for the content and writing of this article. This study was supported by the Environmental Research and Technology Development Fund (C-1154) of the Japanese Ministry of the Environment.

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Research Article

Decreased Pulmonary Function in School Children in Western Japan after Exposures to Asian Desert Dusts and Its Association with Interleukin-8

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The objective of the study was to investigate the influence of Asian dust storms (ADS) on pulmonary function of school children and the relationship of this effect with interleukin-8. Morning peak expiratory flow (PEF) was measured daily in 399 children from April to May 2012 and in 384 of these children from March to May 2013. The data were analyzed for an association between ADS events and PEF by linear mixed models. Interleukin-8 transcriptional activity was assessed in THP-G8 cells stimulated by airborne particles collected on ADS days. Seven ADS days were identified: April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013. Changes in PEF after ADS exposure were −8.17 L/min (95% confidence interval, −11.40 to −4.93) in 2012 and −1.17 L/min (−4.07 to 1.74) in 2013, and there was a significant difference between 2012 and 2013. Interleukin-8 transcriptional activity was significantly higher in 2012 at 10.6 ± 2.9-fold compared to 3.7 ± 0.4 in March 8 to 10, 2013, and 2.3 ± 0.2 in March 19 and 20, 2013. The influence of ADS events on pulmonary function of children differs with each ADS event and may be related to interleukin-8 production.

1. Introduction

Asian dust storms (ADS) originating in the deserts of Mongolia, Northern China, and Kazakhstan often disperses dust over East Asia from spring until late autumn and is the second strongest source of dust emission worldwide [1]. An ADS is also a source of air pollutants because the dust contains chemicals, contaminating metals, microorganisms, and ionic components [2–4]. Therefore, ADS is a serious health problem associated with heavy pollution.

Numerous epidemiologic studies have shown that exposure to ADS increases rates of mortality, emergency treatment, and hospitalization for cardiovascular disease and pulmonary disease [5–8]. Other studies have shown that ADS increases the risk of hospitalization and exacerbates pulmonary function and respiratory symptoms in patients with asthma in Japan and South Korea [9, 10]. However, some studies from Taiwan have suggested that there is no significant association of ADS with asthma [11, 12]. Therefore, the influence of ADS on asthma may differ in different regions. This may be associated with differences in the materials attached to ADS airborne particles, which is influenced by the path the particles take [2–4]. We have also shown that effects on lower respiratory tract symptoms in adult patients with asthma differ for each ADS event [13]. Onishi et al. suggested that ADS events can be classified into three types

based on Lidar data: Type 1 events with high counts of air pollution aerosols, Type 2 events with high counts of mineral dust particles, in comparison to air pollution aerosols, and Type 3 events with very low counts of air pollution aerosols [4].

Neutrophils migrate to the lung during acute inflammation induced by exposure to air pollutants [14]. The concentration of interleukin-8 (IL-8) in bronchial lavage fluid and IL-8 mRNA expression in bronchial biopsy tissue from healthy subjects are also increased by air pollutants [15]. IL-8 is increased in the blood of asthma patients during exacerbation [16] and thus is thought to be a key cytokine in exacerbation of asthma. In this context, we found that airborne particles collected on ADS days in Western Japan induced production of IL-8 in THP-G8 cells, whereas this effect did not occur with the original soil of the ADS [17].

In 2012, a study was already conducted to investigate the influence of ADS and air pollutants on pulmonary function of school children in Western Japan. In the current study, to investigate the difference of the influence of ADS events on pulmonary function in children, we conducted an extended survey in 2013, which was to monitor daily peak expiratory flow (PEF) in the same children as the 2012 investigation. Each year, using an IL-8 luciferase assay, these related detrimental effects on pulmonary function and differences in IL-8 promoter activity induced by ADS airborne particles were studied.

2. Materials and Methods

2.1. Subjects. The aim of this longitudinal follow-up study was to examine effects of ADS events on pulmonary function in school children. Daily morning PEF of children was monitored from March to May 2012 and 2013 because ADS events are most frequent in these months. March 2012 was used as trial period to allow the children to familiarize themselves with the monitoring. There was no ADS event in March 2012. The study was performed in Matsue, the capital city of Shimane Prefecture, in Southwest Japan. The population of Matsue is about 200,000 and the area is 530.2 km^2. In March 2012, all 401 fourth grade students aged 8 to 9 years from 4 of 35 elementary schools in Matsue were enrolled in the study. The four elementary schools were within 10 km of each other and all subjects lived within a radius of 1 km of the schools.

The disposition of the children in the study is shown in Figure 1. A total of 401 children were recruited into the study in March 2012. Two were subsequently excluded due to failure to keep a daily record for PEF. Thus, records of daily PEF were analyzed for 399 children in 2012. In March 2013, we recruited the same 401 children, of whom one was excluded due to Moyamoya disease. Sixteen children were subsequently excluded due to failure to keep a daily record for PEF. Thus, records of daily PEF were analyzed for 384 children in 2013.

The subjects recorded their age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergy in March 2012 and March 2013. Subjects were defined as having asthma if they met any of the following criteria in the past 12 months: (1) diagnosis of asthma by a pediatrician, (2) wheezing, (3) use of asthma medication, and (4) visiting a hospital regularly

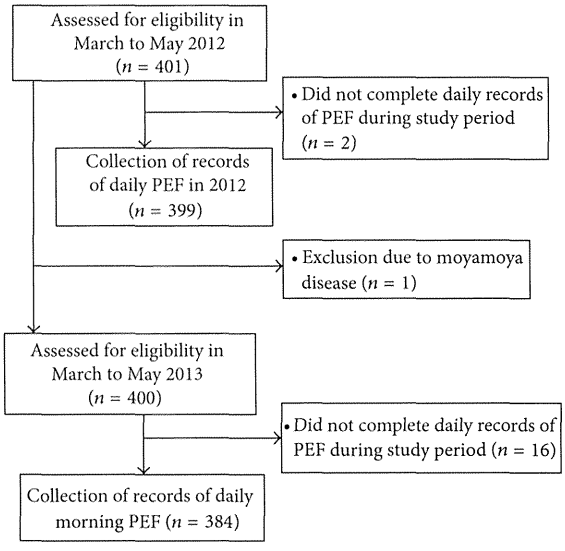


FIGURE 1: Flow chart showing the disposition of children in the study.

for asthma. Similarly, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergy were judged to be present if the subjects met any of the following criteria in the past year: (1) diagnosis by a pediatrician, (2) use of medication for the disease, and (3) visiting a hospital regularly for the disease.

The study was approved by the institutional ethics committee (Ethics Committee of Tottori University, approval number 1764). We asked the Matsue City Board of Education for their help and received approval to submit the study to the schools. The study was also approved by the Parent Teacher Association (PTA) of each elementary school. Children and their parents were informed by teachers and we obtained formal consent for the study from the Matsue City Board of Education.

2.2. Monitoring of PEF. Before the study, the children and teachers were taught how to measure PEF. All children then measured their morning PEF daily using a peak flow meter (Mini-Wright, Harlow, England, American Thoracic Society Scale) from March to May 2012 and 2013, except for weekends and public holidays. Children recorded their best PEF value from three attempts after arriving at school between 8 a.m. and 9 a.m.

2.3. Definition of ADS Days and Air Pollutant Monitoring. The Japan Meteorological Agency has observatories throughout Japan and defines an ADS day based on a criterion of visibility <10 km due to dust arising from the deserts of East Asia, as determined by meteorological satellites monitoring each area. In this study, we used data from the Matsue observatory and we also referred to data from Light Detection and Ranging (Lidar) to define an ADS day. Lidar depolarization measurements performed simultaneously at two wavelengths can be used to identify nonspherical dust particles, which are mineral dust particles, and spherical aerosols such as organic

aerosols and inorganic sulfates and nitrates [19, 20]. Thus, Lidar can be used to measure levels of mineral dust particles as airborne sand dust particles and nonmineral dust particles as air pollution aerosols in real time. Lidar data are collected continuously in 23 locations in Japan, South Korea, China, Mongolia, and Thailand to detect a potential ADS. Other studies have defined ADS events as a daily (24-hour) average of mineral dust particles $>0.1\,km^{-1}$ [9] or $0.066\,km^{-1}$ (moderate ADS day) and $0.105\,km^{-1}$ (heavy ADS day) [21].

2.4. Preparation of Airborne Particles Collected on ADS Days. On ADS days, dust particles were collected at Tottori Prefectural Institute of Health and Environment, Yurihama, Tottori, which is located 70 km east of Matsue. This is the most suitable area close to Matsue to monitor particulate matter from East Asia because Yurihama is rural and has no source of air pollutants, except for motor vehicles. The observatory in Yurihama is also located away from populated areas. ADS airborne particles were collected in Tottori on April 23 and April 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013, using a high-volume air sampler (HV-1000R, Shibata, Tokyo, Japan) on the roof of a building. ADS airborne particles were filtered based on their aerodynamic diameters (Andersen Sampler, Shibata) into 5 sizes (<1.1, 1.1–2.0, 2.0–3.3, 3.3–7.0, and >7.0 μm) and each filter was dried in a desiccator before and after sampling to be weighed. ADS airborne particles of 3.3–7.0 μm were subsequently used in the study. Collected airborne dust was sterilized at 121°C for 30 min in an autoclave (Tomy SX-300, Tomy, Tokyo, Japan) to prevent growth of bacteria and fungi and dried at 80°C for 4 h with drying sterilizer (SG600, Yamato Scientific, Tokyo, Japan). The collected airborne dust was then weighed and stored in a freezer at −20°C. Soil from the China Loess Plateau (CJ-1), the original ADS soil from the Tengger Desert and Huining County located in Gansu Province, was obtained from the National Institute for Environmental Studies (Ibaraki, Japan) in 2002. This reference material is certified by the National Institute for Environmental Studies and the National Research Center for Environmental Analysis and Measurement (Beijing, China). To stimulate THP-G8 cells, airborne particles collected on ADS days were diluted to 1 mg/mL with distilled deionized water.

2.5. IL-8 Promoter-Luciferase Gene Reporter Assay and Measurements of IL-8 and Endotoxin. THP-G8 cells are a THP-1-derived reporter cell line that express stable luciferase orange (SLO) and stable luciferase red (SLR) genes under control of the IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters, respectively [22]. The THP-G8 cell line was kindly provided by the Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan, and was cultured as described previously [22]. We first stimulated THP-G8 cells (5×10^4 cells/100 μL/well) in 96-well black plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with lipopolysaccharide (LPS) (Wako Pure Chemicals, Osaka, Japan) and examined IL-8 and GAPDH reporter activity for various time periods and concentrations. Luciferase activity was determined using a microplate luminometer with a Phelios multicolor detection system (Atto, Tokyo, Japan) using Tripluc luciferase assay reagent (Toyobo, Osaka, Japan). IL-8 transcriptional activity was assessed from normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA, and the fold induction of nSLO-LA was calculated as the nSLO-LA level of treated cells divided by that of untreated cells [22]. Induction of IL-8 transcriptional activity was measured after THP-G8 cells were stimulated for 5 h with solvent only (negative control), 100 ng/mL lipopolysaccharide (LPS), and ADS airborne particles collected in 2012 and 2013.

IL-8 concentrations in culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit for IL-8 (R&D Systems, Minneapolis, MN, USA). Samples were run in triplicate and read using an automated ELISA reader (Model 680, Bio-Rad, Philadelphia, PA, USA). The range of the assay was 31.2 to 2000 pg/mL. Endotoxin concentrations in ADS airborne particles were measured using a chromogenic LAL endotoxin assay kit (GenScript, Piscataway, NJ, USA). The range of the assay was 0.01 to 1 EU/mL. The pH of ADS airborne particles was measured with a pH meter (MP220, Mettler Toledo, Schwerzenbach, Switzerland).

2.6. Measurement of Metal Elements in CJ-1 Soil and ADS Airborne Particles. Metal elements in CJ-1 soil and ADS airborne particles collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 were measured by Oki Engineering (Tokyo, Japan). The concentrations of aluminum (Al), arsenic (As), barium (Ba), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), potassium (K), lanthanum (La), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), strontium (Sr), titanium (Ti), and zinc (Zn) were measured by inductively coupled plasma atomic emission spectrometry. Silicon (Si) was measured using electrothermal atomic absorption spectrometry.

2.7. Statistical Analysis. To evaluate the effects of exposure to an ADS on the daily PEF of children, linear mixed models that accounted for correlations among repeated measurements within a subject were used to estimate the effects of exposure to an ADS on the daily PEF of children in April to May 2012 and March to May 2013 [23, 24]. Additionally, to adjust for potential confounding factors, we used linear mixed models with the following general form:

$$Y_{ij} = \beta_0 + \beta_1 x_{1,j} + \sum_{k=1}^{p} \beta_k x_{k,ij} + b_{0,i} + \varepsilon_{ij}. \quad (1)$$

Y_{ij} corresponds to the daily PEF for the ith child at the jth day ($i = 1, 2, \ldots, N$; $j = 1, 2, \ldots, T$). $x_{1,j}$ is an exposure variable of jth day (measurement of air pollution), and $x_{k,ij}$ ($k = 2, 3, \ldots, p$) are potential confounding factors involving individual characteristics (age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies) and meteorological variables such as daily temperature, humidity, and atmospheric pressure. $\beta_0, \beta_1, \ldots, \beta_p$ are corresponding fixed effects coefficients, and $b_{0,i}$ is the random effect of intercept

Table 1: Characteristics of children.

	2012	2013
Number	399	384
Gender (male/female)	205/194	194/190
Height (cm)	132.3 ± 5.9	137.7 ± 7.0
Male	132.2 ± 5.5	136.9 ± 6.3
Female	132.4 ± 6.4	138.5 ± 7.7
Weight (kg)	29.5 ± 5.8	32.4 ± 6.6
Male	29.6 ± 6.2	32.3 ± 6.8
Female	29.3 ± 5.4	32.6 ± 6.4
Allergic disease		
Asthma	38	45
Allergic rhinitis	78	74
Allergic conjunctivitis	8	15
Atopic dermatitis	44	36
Food allergy	19	20

Data are shown as the mean ± S.D.

for ith child and are assumed to be $b_{0,i} \sim N(0, \sigma_b^2)$. ε_{ij} is the error term, $\varepsilon_{ij} \sim N(0, \sigma^2)$. In addition, effects on PEF were measured from the day of ADS exposure until 3 days after exposure because a dust effect on PEF can persist for up to 3 days [10]. Differences in PEF between the 2012 and 2013 results were also evaluated. The two-pollutant model was applied to different combinations of pollutants to assess the stability of the effects of ADS on PEF after adjustment for individual characteristics (age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies) and meteorological variables (temperature, humidity, and atmospheric pressure). R version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis of PEF values and ADS exposure. Differences of nSLO-LA of THP-G8 cells were analyzed by ANOVA using SPSS Statistics (Japanese version 21.0 for Windows, IBM Japan, Tokyo, Japan). All quoted P values are two-sided and the significance level was set to 0.05.

3. Results

3.1. Profile of the Children. The characteristics of the children in the 2012 and 2013 studies are shown in Table 1.

3.2. Air Pollution Levels and Weather Information on ADS Days and Non-ADS Days. In 2012, April 23 and 24 were identified as ADS days. In 2013, March 8 to 10 and 19 and 20 were similarly identified as ADS days. Non-ADS days were defined as all other days from April 1 to May 31, 2012, and from March 1 to May 31, 2013. Daily levels of mineral dust particles (airborne sand dust particles) and suspended particulate matter (SPM) are shown in each period in Figure 2. The levels of air pollutants and weather during the study periods are shown in Table 2.

3.3. PEF. Changes in PEF after exposure to ADS are shown in Figure 3. In order to show the post-ADS-exposure effects,

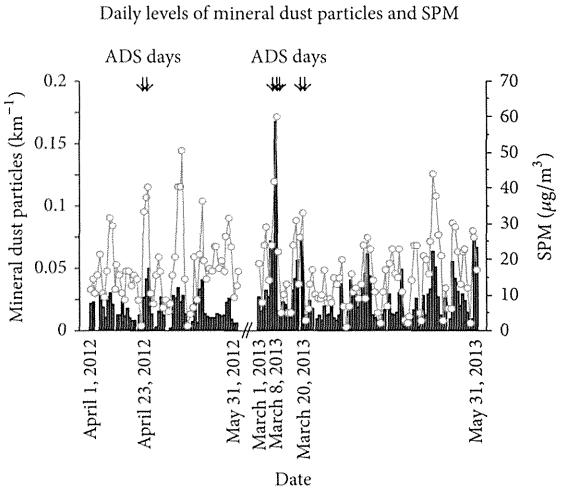


Figure 2: Daily levels of mineral dust particles (airborne sand dust particles) (bar graph) and SPM (line graph). Arrows indicate ADS days.

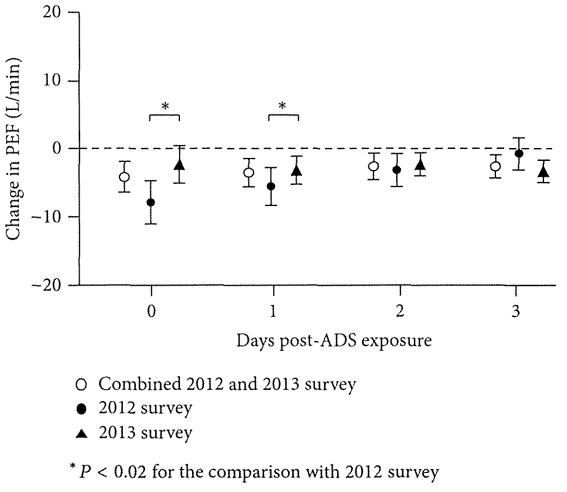


Figure 3: PEF changes caused by an ADS event from 0 (ADS day) to 3 days after ADS exposure in combined 2012 and 2013 (open circles), 2012 (black circles), and 2013 (triangles), with 95% confidence intervals (error bars). Data are controlled for age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies; meteorological variables such as daily temperature, humidity, and atmospheric pressure; and the linear time trend. There are significant differences in the decrement of PEF on days 0 and 1 between 2012 and 2013 ($^*P < 0.02$).

these changes are shown from 0 (ADS day) to 3 days after ADS exposure. In combining 2012 and 2013, the changes in PEF after exposure to ADS exposure were −4.16 L/min (95% CI, −6.33 to −1.99) on day 0, −2.97 L/min (−5.03 to −0.91) on day 1, −2.23 L/min (−4.12 to −0.35) on day 2, and −2.57 L/min (−4.29 to −0.86) on day 3 after ADS. There were significant decreases in PEF from day 0 to day 3 after ADS exposure. In 2012, the changes in PEF were −7.82 L/min (−10.93 to −4.71)

Table 2: Daily air pollutant levels and weather information on ADS days and non-ADS days in 2012 and 2013.

(a)

Measurement	ADS days		
	April 23 and 24, 2012	March 8 to 10, 2013	March 19 and 20, 2013
Daily average temperature, °C	17.6 ± 1.1	13.6 ± 3.8	12.4 ± 1.4
Daily maximum temperature, °C	23.3 ± 3.0	20.4 ± 0.9	18.6 ± 0.0
Daily minimum temperature, °C	13.1 ± 0.7	8.0 ± 5.8	7.9 ± 4.5
Daily average relative humidity, %	70.0 ± 5.7	64.7 ± 7.6	79.5 ± 3.5
Daily minimum relative humidity, %	48.0 ± 12.7	29.3 ± 10.7	52.0 ± 0.0
Daily average atmospheric pressure, hPa	1010.8 ± 1.0	1008.6 ± 3.1	1007.4 ± 3.0
Daily average mineral dust particles, km^{-1}	0.046 ± 0.006	0.084 ± 0.077	0.075 ± 0.001
Daily average nonmineral dust particles, km^{-1}	0.148 ± 0.097	0.138 ± 0.050	0.097 ± 0.001
Daily average SPM, $\mu g/m^3$	39.5 ± 2.1	44.3 ± 19.0	32.5 ± 5.0
Daily average $PM_{2.5}$, $\mu g/m^3$	17.2 ± 1.3	37.3 ± 16.7	37.8 ± 4.5
Daily average SO_2, ppb	1.3 ± 0.6	2.0 ± 1.1	1.9 ± 1.3
Daily average NO_2, ppb	1.5 ± 0.4	3.4 ± 1.8	3.6 ± 0.6
Daily average O_x, ppb	55.9 ± 5.7	63.1 ± 10.5	49.4 ± 5.3

(b)

Measurement	Non-ADS days	
	2012	2013
Daily average temperature, °C	15.7 ± 3.5	13.2 ± 5.0
Daily maximum temperature, °C	20.9 ± 4.5	18.7 ± 5.7
Daily minimum temperature, °C	10.9 ± 4.0	8.1 ± 5.0
Daily average relative humidity, %	70.4 ± 9.3	69.8 ± 9.8
Daily minimum relative humidity, %	44.9 ± 15.4	42.8 ± 13.6
Daily average atmospheric pressure, hPa	1010.0 ± 5.7	1011.3 ± 5.9
Daily average mineral dust particles, km^{-1}	0.016 ± 0.010	0.024 ± 0.017
Daily average non-mineral dust particles, km^{-1}	0.042 ± 0.037	0.073 ± 0.049
Daily average SPM, $\mu g/m^3$	17.7 ± 10.1	17.8 ± 8.9
Daily average $PM_{2.5}$, $\mu g/m^3$	10.3 ± 5.4	17.5 ± 7.3
Daily average SO_2, ppb	0.9 ± 0.6	1.0 ± 0.8
Daily average NO_2, ppb	2.6 ± 1.2	2.75 ± 1.2
Daily average O_x, ppb	50.0 ± 7.5	50.2 ± 8.4

Data are presented as the mean ± S.D., Non-ADS days were all other days except for ADS days from April 1 to May 31, 2012, and March 1 to May 31, 2013.

on day 0, −5.49 L/min (−8.14 to −2.85) on day 1, −3.15 L/min (−5.54 to −0.75) on day 2, and −0.72 L/min (−3.03 to 1.59) on day 3 after ADS. A significant decrease in PEF persisted for 2 days after ADS exposure in 2012. In 2013, the changes in PEF were −2.33 L/min (−5.09 to 0.44) on day 0, −2.72 L/min (−4.80 to −0.64) on day 1, −2.26 L/min (−3.98 to −0.53) on day 2, and −3.04 L/min (−4.68 to −1.40) on day 3 after ADS. A significant decrease in PEF continued from days 1 to 3 after ADS exposure. On days 0 and 1, the decrease in PEF after exposure to ADS in 2012 was significantly higher than that in 2013. In addition, the 2012 and 2013 forest plots indicate clear differences. Significant differences were observed in PEF on days 0 and 1, and the decrement of PEF in 2012 was higher than that in 2013. In a two-pollutant model adjusted for SPM, $PM_{2.5}$, SO_2, NO_2, and O_x, an ADS event in 2012 alone was significantly associated with a decrease of PEF (Table 3). In contrast, in 2013, a similar model gave no significant relationship between ADS events and PEF in children.

3.4. IL-8 Transcriptional Activity and IL-8 Secretion in THP-G8 Cells. In THP-G8 cells stimulated for 5 h with various LPS concentrations, nSLO-LA (a measure of IL-8 transcriptional activity) reached a plateau at 100 ng/mL LPS (Figure 4(a)). Maximum induction of nSLO-LA by LPS (100 ng/mL) occurred between 4 and 6 h (Figure 4(b)). Based on these results, we subsequently used stimulation for 5 h to investigate the effect of ADS airborne particles on IL-8 transcriptional activity. The concentrations of IL-8 in supernatants of THP-G8 cells stimulated with vehicle, LPS (n = 6, 1 ng/mL), and LPS (n = 6, 100 ng/mL) were 1.2 ± 0.2, 26.6 ± 6.2, and 77.4 ± 10.9 μg/mL, respectively (Figure 4(c)). This increase in IL-8 secretion is in agreement with the augmentation of nSLO in THP-G8 cells.

The pH values of ADS airborne particles (1 mg/mL) collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 were 7.9, 7.6, and 7.6, respectively. The nSLO-LA values (IL-8 transcriptional activity) of THP-G8

Table 3: Estimated effects of ADS events on PEF in two-pollutant model after adjustment for SPM, $PM_{2.5}$, NO_2, O_x, and SO_2.

Year	Adjustment	Change in PEF	95% CI	*P* value
2012 and 2013	Adjusted for SPM	−3.00	−5.31, −0.68	0.011
	Adjusted for $PM_{2.5}$	−3.60	−5.94, −1.27	0.002
	Adjusted for SO_2	−2.14	−4.43, 0.15	0.059
	Adjusted for O_x	−3.49	−5.70, −1.28	0.002
	Adjusted for NO_2	−4.20	−6.37, −2.03	0.001
2012	Adjusted for SPM	−6.04	−9.44, −2.64	0.001
	Adjusted for $PM_{2.5}$	−6.48	−9.78, −3.18	0.001
	Adjusted for SO_2	−7.41	−10.69, −4.13	0.001
	Adjusted for O_x	−3.93	−7.25, −0.62	0.019
	Adjusted for NO_2	−10.04	−13.42, −6.67	0.001
2013	Adjusted for SPM	−1.57	−4.56, 1.43	0.306
	Adjusted for $PM_{2.5}$	−1.97	−5.10, 1.15	0.216
	Adjusted for SO_2	−2.19	−5.02, 0.63	0.128
	Adjusted for O_x	0.19	−2.79, 3.18	0.900
	Adjusted for NO_2	−2.45	−4.38, 1.49	0.085

Calculated for an interquartile by ADS and adjusted for individual characteristics (age, gender, height, weight, and presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies) and meteorological variables (temperature, humidity, and atmospheric pressure).
ADS: Asian dust storm, PEF: peak expiratory flow, SPM ($\mu g/m^3$): suspended particle matter, $PM_{2.5}$ ($\mu g/m^3$): particulate matter smaller than 2.5 μm in diameter, NO_2 (ppb): nitrogen dioxide, O_x (ppb): photochemical oxidants, SO_2 (ppb): sulfur dioxide, and CI: confidence interval.

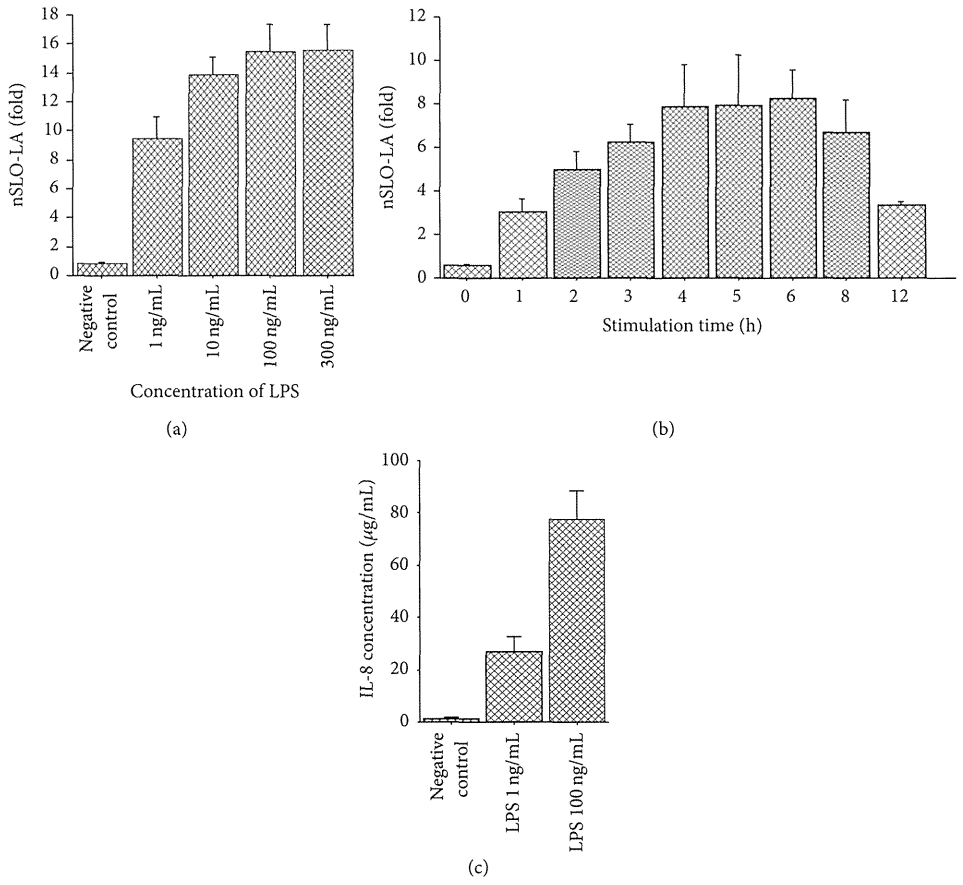
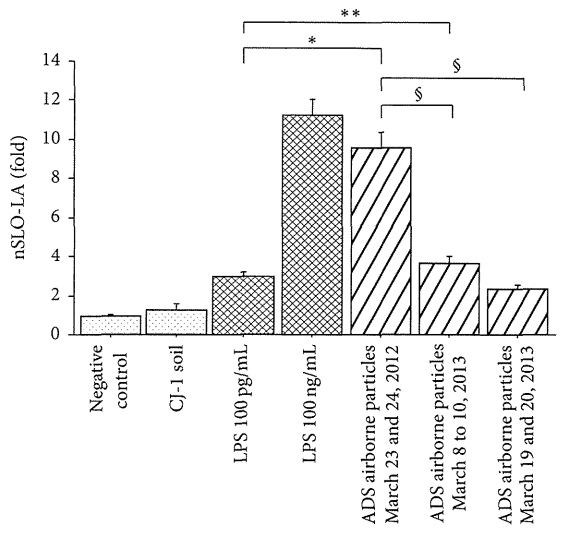


Figure 4: (a) IL-8 transcriptional activity in THP-G8 cells stimulated with LPS at various concentrations ($n = 6$) for 5 h. IL-8 transcriptional activity is based on normalized SLO luciferase activity (nSLO-LA), which was calculated as SLO-LA divided by SLR-LA. The fold induction of nSLO-LA was calculated as the nSLO-LA of treated cells divided by that of untreated cells [18]. (b) IL-8 transcriptional activity in THP-G8 cells stimulated with 100 ng/mL LPS ($n = 6$) for various time periods. (c) Concentrations of IL-8 in supernatants of a stable THP-1-derived IL-8 reporter cell line stimulated with solvent only (negative control), LPS ($n = 6$, 1 ng/mL), and LPS ($n = 6$, 100 ng/mL). The IL-8 concentration was measured using an ELISA kit. Samples were run in triplicate. The assay range was 31.2 to 2000 pg/mL.



*$P < 0.01$ for the comparison with LPS at 100 pg/mL

**$P < 0.02$ for the comparison with LPS at 100 pg/mL

$^{\S}P < 0.01$ for the comparison with ADS airborne particles in April 23 and 24, 2012

FIGURE 5: IL-8 transcriptional activity measured using an IL-8 luciferase assay in a stable THP-1-derived IL-8 reporter cell line. Cells were treated with solvent only ($n = 6$, negative control), LPS ($n = 6$, 100 pg/mL, positive control), LPS ($n = 6$, 100 ng/mL, positive control), and ADS airborne particles collected on April 23 and 24, 2012 ($n = 6$, 1 mg/mL), March 8 to 10, 2013 ($n = 6$, 1 mg/mL), and March 19 and 20, 2013 ($n = 6$, 1 mg/mL). *$P < 0.01$ versus LPS at 100 pg/mL, **$P < 0.02$ versus LPS at 100 pg/mL, and $^{\S}P < 0.01$ versus ADS airborne particles from April 23 to April 24, 2012.

cells (Figure 5) changed by 0.95 ± 0.09-fold (vehicle, $n = 6$), 2.87 ± 0.28-fold (LPS, $n = 6$, 100 pg/mL), 11.21 ± 0.28-fold (LPS, $n = 6$, 100 ng/mL), 9.56 ± 0.80-fold (ADS particles from April 23 to 24, 2012, $n = 6$, 1 mg/mL), 3.65 ± 0.36-fold (ADS particles from March 8 to 10, 2013, $n = 6$, 1 mg/mL), and 2.33 ± 0.24-fold (ADS particles from March 19 to 20, 2013, $n = 6$, 1 mg/mL).

The pH value of CJ-1 soil was constant at 8.4 for each event. The pH values of ADS airborne particles (1 mg/mL) collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 were 7.9, 7.6, and 7.6, respectively. THP-G8 cells were stimulated with CJ-1 soil after adjusting the pH of the soil to 7.8 with 0.1 N sodium hydroxide. The nSLO-LA values (IL-8 transcriptional activity) of THP-G8 cells (Figure 5) changed by 0.95 ± 0.09-fold (vehicle, $n = 6$), 1.48 ± 0.27-fold (CJ-1 soil, $n = 6$, 1 mg/mL), 2.87 ± 0.28-fold (LPS, $n = 6$, 100 pg/mL), 11.21 ± 0.28-fold (LPS, $n = 6$, 100 ng/mL), 9.56 ± 0.80-fold (ADS particles from April 23 to 24, 2012, $n = 6$, 1 mg/mL), 3.65 ± 0.36-fold (ADS particles from March 8 to 10, 2013, $n = 6$, 1 mg/mL), and 2.33 ± 0.24-fold (ADS particles from March 19 to 20, 2013, $n = 6$, 1 mg/mL). nSLO-LA values in THP-G8 cells stimulated by ADS airborne particles differed significantly from those of controls and cells stimulated with 100 pg/mL LPS. nSLO-LA values also differed significantly for each pairwise comparison of airborne particles collected in the three ADS periods.

3.5. Endotoxin Concentration in Airborne Particles Collected on ADS Days. The endotoxin levels in ADS airborne particles (1 mg/mL) collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 were 0.19, 0.08, and 0.07 EU/mL, respectively. These values were all lower than the level of 0.89 EU/mL found in 100 pg/mL LPS. The endotoxin concentration in 100 ng/mL LPS was out of the range of the assay.

3.6. Concentration of Metal Elements in CJ-1 Soil and Airborne Particles Collected on ADS Days. The concentrations of metal elements in CJ-1 soil and ADS airborne particles collected on April 23 and 24, 2012; March 8 to 10, 2013; and March 19 and 20, 2013 are shown in Table 4.

4. Discussion

To investigate the effect of ADS on pulmonary function, we monitored daily PEF in school children from April to May 2012 and March to May 2013 and found a significant correlation between exposure to an ADS and pulmonary function. The 2012 survey alone also showed this relationship. When differences in PEF between the 2012 and 2013 results were evaluated, the same relationship of PEF with ADS events was not found in 2013, despite the study being conducted in the same children. The decline of PEF upon ADS exposure in 2012 was also significantly higher than that in 2013, and IL-8 transcriptional activity in THP-G8 cells induced by ADS airborne particles collected in 2012 was also significantly higher than that induced by ADS airborne particles collected in 2013. These results suggest that the effect of ADS on pulmonary function in children is associated with enhanced airway inflammation mediated by elevation of IL-8.

Desert sand can reduce pulmonary function in patients with asthma after exposure at a level of PM_{10} ranging from 1500 to 2000 $\mu g/m^3$/hour [25]. This level of PM_{10} is 10 to 20 times higher than that during an ADS event in Japan. In the current study, the level of mineral dust particles on ADS events on March 8 to 10, 2013, and March 19 and 20, 2013 was about twice as high as that in 2012. However, the decrease of PEF after exposure to ADS in 2012 was higher than that in 2013. These results suggest that the decline in pulmonary function of school children during an ADS event has little connection to mineral dust particles (sand dust particles). Nonmineral dust particles were similar on ADS events in 2012 and 2013. ADS events in 2012 had higher counts of air pollution aerosols than those in 2013. Based on Onishi's criteria [4], the ADS event in 2012 can be classified as Type 1 and those in 2013 as type 2. The air pollution aerosols during ADS can be considered a cause of the significant difference in the effect of the ADS on pulmonary function of school children between 2012 and 2013.

The ADS days in 2013 were defined as moderate, while, on April 23 and 24, 2012, the mean daily average concentration of mineral dust particles was 0.046 ± 0.006 km^{-1} in Matsue

Table 4: Concentration of metal elements in CJ-1 soil and airborne particles collected on ADS days.

Metals (μg/mg)	CJ-1 soil	ADS airborne particles in April 23 and 24, 2012	ADS airborne particles in March 8 to 10, 2013	ADS airborne particles in March 19 and 20, 2013
Al	68.00	28.80	22.40	14.80
As	ND	ND	ND	ND
Ba	0.44	0.12	0.18	0.10
Ca	68.00	29.60	44.00	31.20
Cd	ND	ND	ND	ND
Co	0.01	ND	ND	ND
Cr	0.05	ND	ND	ND
Cu	0.03	0.12	ND	0.07
Fe	26.00	22.00	20.80	14.40
Hg	ND	ND	ND	ND
K	0.19	0.38	0.33	0.30
La	0.03	ND	ND	ND
Mg	18.00	14.00	16.80	14.40
Mn	0.70	0.52	0.56	0.40
Na	0.44	33.60	56.00	64.00
Ni	0.03	0.14	0.15	0.11
P	0.68	ND	ND	ND
Pb	0.02	0.06	0.12	0.06
Si	260.00	140.00	108.00	72.00
Sr	0.26	0.16	0.22	0.16
Ti	2.40	0.96	0.92	0.52
Zn	0.07	0.52	0.64	0.60

ADS: Asian dust storm, CJ-1 soil: soil from the China Loess Plateau, the original ADS soil in the Tengger Desert and Huining located in Gansu Province, and ND: not detected.

City. These values are lower than the thresholds defined in previous studies [9, 21]. However, the daily average level of mineral dust particles on ADS days was higher than that on non-ADS days, as required for definition of an ADS day in the Japan Meteorological Agency criteria used in this study.

Many studies have shown that children are susceptible to air pollution such as NO_2, O_x, and SO_2 [18, 26]. Therefore, we used a linear mixed model and a two-pollutant model to adjust for the effects of NO_2, O_x, and SO_2 on pulmonary function. In both models, ADS in 2012 remained significant after inclusion of NO_2, O_x, and SO_2. However, in the 2013 survey, we were not able to find a significant association with ADS and pulmonary function. These results suggest that airborne particles during ADS decrease pulmonary function irrespective of NO_2, O_x, and SO_2.

IL-8 is a key cytokine in air pollutant-induced airway inflammation [15, 16]. Components that adhered to ADS particles can increase release of IL-6 and IL-8 from airway epithelial cells [27]. We showed that ADS airborne particles promote transcriptional activity and production of IL-8 in THP-G8 cells, with a significant increase in IL-8 transcriptional activity in THP-G8 cells treated with ADS airborne particles compared to those treated with original ADS soil (CJ-1 soil). Additionally, we measured the difference in production of IL-8 by particles collected during each ADS event. The IL-8 transcriptional activity of ADS airborne particles collected in 2012 was significantly higher than that for particles collected in 2013. This difference in production of IL-8 by ADS airborne particles may account for the different effects on pulmonary function in school children in 2012 and 2013.

The production of IL-8 induced by ADS airborne particles in 2012 had a significant difference compared to 2013. However, there was no difference between the two ADS airborne particles in 2013. The ADS events in 2013 had happened close together, and we suspect that the route and composition of the ADS airborne particles in the two events were similar. In fact, when we analyzed the effect on PEF between two ADS events in 2013 separately, the decreases in PEF after exposure to ADS were −4.1 L/min (95% CI, −10.6 to 2.4, $P = 0.21$) in March 8 to 10 and −3.3 L/min (95% CI, −10.8 to 4.1, $P = 0.37$) in March 19 and 20. In both ADS events in 2013, there was not a significant decrease of the effects on PEF. Therefore, we presented the combined results in the main analysis. The differences in the substances and the levels of those substances attached to desert sand dusts depend on the route along which desert sand dusts pass and may play an important role in the effect of ADS on pulmonary function in children.

According to the analysis of the concentrations of metal elements, ADS airborne particles in 2012 had more Al, Cu, Fe, K, and Ti compared to those on March 8 to 10 and March 19 and 20, 2013. The amounts of Al, Fe, and Ti in ADS airborne particles in 2012 were lower than CJ-1. Cu and K may play

a causative role in the difference of production of IL-8 induced by ADS airborne particles. However, Kumar et al. indicated that Cu was not a cause of the production of CXCL1 (a mouse functional homologue of IL-8) and IL-6 induced by ambient and traffic-derived particulate matter, but it did indicate that Fe content of airborne particulate matter may be more important in mouse airway epithelial injury [28]. Metal components attached to ADS airborne particles may be one of the causes of the difference between 2012 and 2013, but further study is needed to determine a role of metal components attached to ADS on the effect of production of proinflammatory cytokines.

Ogino et al. found that some proteins contained in ambient particulate matter are important environmental factors that aggravate airway hyperresponsiveness and airway inflammation in mice [29]. An ADS contains different amounts of β-glucan, which can induce airway inflammation [30, 31]. Thus, in addition to chemical substances, anthropogenic metal components, and sulfate, some proteins and β-glucan attached to ADS airborne particles may play important roles in the reduction of pulmonary function during ADS events.

Inhaled LPS is associated with airway neutrophil inflammation in patients with asthma and in healthy subjects [32–34]. Our results show that ADS airborne particles contain endotoxin, and the endotoxin concentration of ADS airborne particle was lower than that in LPS at 100 pg/mL. However, the IL-8 transcriptional activity induced by ADS airborne particle collected on April 23 and 24, 2012 and March 8 to 10, 2013 was significantly higher than that induced by LPS at 100 pg/mL. Endotoxin may augment IL-8 transcriptional activity in THP-G8 cells, in addition to other substances on ADS airborne particles that may induce IL-8.

Park et al. [10] and Yoo et al. [35] found a relationship between ADS events and PEF in Korean children with asthma, while Hong et al. did not find a significant relationship between ADS events and PEF in children without asthma [36]. Patients with allergic diseases may also be more sensitive to air pollution [37–39]. Therefore, in this study, we analyzed the data after adjustment for allergic diseases. This analysis showed that there was a significant decrease of PEF on ADS days in 2012 compared to 2013, regardless of the presence of allergic diseases. However, the number of subjects with each disease was too small to investigate the association of PEF with ADS. Further studies are needed to define the relationship between ADS and PEF in children with allergic diseases.

In this study, children recorded their PEF value after arriving at school but did not record their PEF value on weekends and public holidays. The ADS days March 9, 10, and 20, 2013 lacked PEF data because they were holidays. However, this intermittent missing data is statistically independent of the ADS events. Thus, it would not cause any serious bias in the results. Although it would raise a reduction of statistical power, the significant associations were still observed in the primary analyses.

There are several limitations in the study. First, we did not investigate diseases other than asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies. Second, we were unable to diagnose asthma based on airway hyperresponsiveness to methacholine and reversible airflow limitation. In this study, some children were considered to have asthma, when in fact their wheezing may have been caused by respiratory tract infection or other diseases. However, wheezing caused by respiratory tract infection and other diseases is more common in children under 6 years old, and that is younger than those in our study [40]. Additionally, it is difficult to distinguish asthma and reactive airway disease based on the present diagnostic criteria. Third, we were unable to measure the individual amount of exposure to ADS. Fourth, we did not analyze the composition of the ADS airborne particles. Therefore, this study was not able to investigate which components of ADS airborne particles played important roles in reduction of pulmonary function during the ADS and which components induce IL-8. Further studies are needed to define these components.

5. Conclusion

We conclude that the effect of exposure to ADS on pulmonary function in school children differed among ADS events, and that enhancement of IL-8 transcriptional activity also differed among ADS airborne particles collected during the respective events. These findings suggest that substances attached to ADS airborne particles exacerbate pulmonary function of school children. Further studies are needed to identify the substances attached to the ADS airborne particles that play key roles in exacerbation of pulmonary function.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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RESEARCH PAPER

Dual-color bioluminescence imaging assay using green- and red-emitting beetle luciferases at subcellular resolution

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Abstract Bioluminescence imaging is widely used to monitor cellular events, including gene expression in vivo and in vitro. Moreover, recent advances in luciferase technology have made possible imaging at the single-cell level. To improve the bioluminescence imaging system, we have developed a dual-color imaging system in which the green-emitting luciferase from a Brazilian click beetle (Emerald Luc, ELuc) and the red-emitting luciferase from a railroad worm (Stable Luciferase Red, SLR) were used as reporters, which were localized to the peroxisome and the nucleus, respectively. We clearly captured simultaneously the subcellular localization of ELuc in the peroxisome and SLR in the nucleus of a single cell using a high-magnification objective lens with 3-min exposure time without binning using a combination of optical filters. Furthermore, to apply this system to quantitative time-lapse imaging, the activation of nuclear factor triggered by tumor necrosis factor α was measured using nuclear-targeted SLR and peroxisome-targeted ELuc as the test and internal control reporters, respectively. We successfully quantified the kinetics of activation of nuclear factor κB using nuclear-targeted SLR and the transcriptional change of the internal control promoter using peroxisome-targeted ELuc simultaneously in a single cell, and showed that the activation kinetics, including activation rate and amplitude, differed among cells. The results demonstrated that this imaging system can visualize the subcellular localization of reporters and track the expressions of two genes simultaneously at subcellular resolution.

Keywords Dual-color bioluminescence imaging · Luciferase · Nuclear factor κB · Subcellular imaging · Time-lapse imaging

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Introduction

Bioluminescent reporters have become an essential tool for studying various aspects of biological functions, including gene expression, posttranscriptional modification, and protein–protein interactions, because the sensitivity and range of the linear response are superior to those of other reporters, including β-galactosidase, chloramphenicol acetyltransferase, and fluorescent proteins [1, 2]. In particular, luciferases are used as sensitive reporters to monitor gene expression noninvasively, quantitatively, and longitudinally in living cells, in explant tissues, and in vivo [3–5].

Recent advances in luciferase technology have made possible the quantitative visualization of gene expression at single-cell resolution by imaging luciferase luminescence in real time using a highly sensitive charged-coupled device (CCD) camera [4–6]. Although fluorescence imaging techniques that use fluorescent proteins (e.g., green fluorescent protein and its derivatives) as probes have contributed immensely to the advancement of cell biology and are used as powerful probes to monitor an extensive array of entities, ranging from single molecules to whole organisms,

bioluminescence imaging is rapidly emerging as a new and sensitive approach to understanding cell physiology.

The luciferase emits light by oxidizing its substrate, luciferin, in a specific manner [7]. Among the possible luciferase/luciferin reactions, the beetle luciferase and D-luciferin (a benzothiazole) pair is the best probe for the long-term and noninvasive detection of cellular events, because the luminescence generated by the reaction is highly quantitative and has an extremely low background, and D-luciferin is stable and easily permeates cells and tissues [8–10]. Moreover, no external illumination is required to induce bioluminescent reactions. Therefore, the characteristic properties of the beetle luciferase/luciferin reaction allow cellular events to be monitored longitudinally and quantitatively.

Of the luciferases identified to date, the firefly luciferase from *Photinus pyralis* is the most commonly used as a bioluminescent reporter. Although this reporter has been extensively used to monitor multiple cellular events in cell extraction assays, cell-based assays, and in vivo and single-cell imaging [1, 2, 11], the light output of this reporter from living cells is insufficient for analyses at high temporal and/or spatial resolution. In particular, bioluminescence imaging at the subcellular level is difficult because of the insufficient light output of available probes. To overcome this limitation, we have recently developed a brighter green-emitting luciferase (Emerald Luc, ELuc), from Brazilian click beetle, and achieved imaging at the subcellular level with high resolution [12].

Improvements in luciferases and detection systems have enabled us to monitor the expressions of multiple genes simultaneously using luciferases that emit light of different colors [4, 5]. To detect two genes simultaneously, green- and red-emitting beetle luciferases that act on a single D-luciferin substrate are commonly used. Any two or more luciferases that stably emit separable emission spectra can be combined to monitor the expression of multiple genes simultaneously. This system has been widely used for cell extraction assays or real-time monitoring in bacteria [13], yeasts [14], cultured plant tissues [15], cultured mammalian cells [16–25], and explanted tissues [26, 27], as well as for in vivo imaging [28–31]. Furthermore, more recently, we and others have applied the dual-color luciferase system to the time-lapse bioluminescence imaging of a single cell to quantitatively and simultaneously track expressions of clock genes [32] and genes involved in somitogenesis [33, 34] using ELuc and a red-emitting luciferase (Stable Luciferase Red, SLR).

Although the dual-color luciferase system has succeeded in visualizing multiple gene expressions simultaneously at the single-cell level, it has never achieved dual-color imaging at subcellular resolution. To improve the bioluminescence imaging system, we have developed a dual-color bioluminescence imaging system in which peroxisome-targeted ELuc and nuclear-targeted SLR are used as reporters in combination with optical filters. With this system, we have successfully imaged the subcellular localization of the reporters simultaneously with high spatiotemporal resolution, and tracked two gene expressions at subcellular resolution.

Materials and methods

Plasmid construction

To construct a reporter plasmid carrying peroxisome-targeted ELuc, the complementary DNA sequence in which the start codon was replaced by an *Eco*RV site was amplified by polymerase chain reaction using pELuc-test (Toyobo, Osaka, Japan) as the template. The amplified product was ligated into the *Eco*RV/*Xho*I site of pCMV-Tag2B (Stratagene) downstream of the immediate cytomegalovirus (CMV) promoter, which resulted in the pCMV-ELuc (pox) construct. To generate plasmid carrying nuclear-targeted SLR, the complementary DNA sequence in which the peroxisomal targeting signal (Ser-Lys-Leu) at the extreme C-terminus and the stop codon of SLR were replaced by a *Xho*I site was amplified by polymerase chain reaction using pSLR-test (Toyobo) as the template. The amplified fragment was ligated into the *Nco*I/*Xho*I site of pCMV/myc/nuc (Invitrogen, Carlsbad, CA, USA), in which a triple nuclear localization signal from SV40 large T antigen was introduced downstream of the multiple-cloning site, for C-terminal fusion to the SLR, resulting in the pCMV-SLR (nuc) construct. To generate a nuclear factor κB (NF-κB) reporter plasmid, oligonucleotides containing six tandem repeats of the NF-κB response element (NF-κB RE; 5′-CGGA AAGTCCA-3′) were ligated into the *Xho*I/*Bgl*II site of pSLR-HSVtk Control (Toyobo), immediately upstream of the herpes simplex virus thymidine kinase promoter. The SLR gene was replaced by nuclear localization signal fused SLR with the *Nco*I and *Xba*I fragment of pCMV-SLR (nuc) to generate pNFκB-TK-SLR (nuc).

Cell culture and transfection procedures

Mouse NIH3T3 cells (Riken Cell Bank 1862) were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS; ICN Biochemicals, Aurora, OH, USA) in a humidified atmosphere containing 5 % CO_2 at 37 °C. One day before the transfection, the cells were seeded in 35-mm glass-bottom dishes (Iwaki, Tokyo, Japan) at 5×10^5 cells per dish unless otherwise noted. Transfection was performed using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions.